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GRANT NUMBER: DAMD17-94-J-4219

TITLE: Effect of HSP27 on Human Breast Tumor Cell Growth and Motility

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REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Annual (1 Sep 95 - 31 Aug 96)

4. TITLE AND SUBTITLE
Effect of HSP27 on Human Breast Tumor Cell Growth and Motility

5. FUNDING NUMBERS
DAMD17-94-J-4219

6. AUTHOR(S)
Eileen Hickey, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Nevada, Reno
Reno, NV 89557-0015

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender. The goal of this research is to investigate the effects of the small stress protein, HSP27, on growth and motility characteristics of normal and tumor-derived human mammary cell lines. Our study is based on the hypothesis that HSP27 is a component of a signal transduction pathway that regulates actin microfilament dynamics. We hypothesize that cells overexpressing HSP27 will show increased motility and altered chemotactic properties, in addition to increased resistance to heat killing and certain drugs. Research completed this year includes optimization of transfection of human MCF7 and MDA231 cells, characterization of sensitivity of mammary cell lines to reagents used in selecting stable transfected cell lines, and selection and assay of clonal cell lines. Donna is introducing the use of adenovirus-mediated transfection, for improved efficiency of transfer of genes into breast cell lines. She has also initiated assays of HSP27 promoter function.

14. SUBJECT TERMS		15. NUMBER OF PAGES
Breast Cancer	stress proteins, hsp27, transfection of mammalian cells	29
		16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Eileen Hickey 9/27/96

PI - Signature / Date

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INTRODUCTION

This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender.

The goal of this research is to investigate the effects of the small stress protein, HSP27, on growth and motility characteristics of normal and tumor derived human mammary cell lines. Preliminary clinical studies indicate that elevated levels of HSP27 in breast tumor cells correlates with aggressive metastasis and poor prognosis (1,2). We have shown that HSP27 overexpression confers resistance to killing by hyperthermia and by certain anti-tumor drugs (3,4). Phosphorylation of HSP27 increases rapidly in cells treated with heat, cytokines or mitogens (5,6,7,8). In rodent cells overexpressing human HSP27, the actin cytoskeleton is resistant to damage caused by hyperthermia or cytochalasin D treatment (9,10). High levels of HSP27 also correlate with increased accumulation of cortical actin, suggesting a possible effect on cellular motility. In contrast, cells expressing a non-phosphorylatable form of HSP27 show inhibition of processes depending on cortical microfilament dynamics (10).

Our study is based on the hypothesis that HSP27 is a component of a signal transduction pathway that regulates actin microfilament dynamics, and may affect cell migration and the metastatic potential of tumors. We propose that cells overexpressing HSP27 will show increased motility and altered chemotactic properties, in addition to increased resistance to heat killing and to certain drugs. Overexpressing cells may respond more vigorously to chemotactic agents, or may respond to different signaling molecules than the parent cell type. We predict that the cells expressing antisense HSP27 sequences, or those expressing the unphosphorylatable mutant will show responses antagonistic to those shown by cells overexpressing normal HSP27.

We proposed to 1) prepare human mammary cell lines expressing either increased levels of HSP27, unphosphorylatable HSP27, or antisense sequences that reduce endogenous HSP27 expression; 2) assay the rate of cell proliferation in these cell lines, compared to controls; 3) assay motility and response to cytokines of these cells using the Boyden chamber technique; and 4) study the resistance of the cell lines to hyperthermia, arsenate, cytochalasin D, and antitumor drugs. These goals would be achieved through the following approaches:

a. Plasmids will be constructed that allow expression of the HSP27 gene independently of estrogen under control of a metallotheionine promoter in human mammary cell lines. Constructions expressing normal HSP27, non-phosphorylatable mutants of HSP27, and antisense sequences for down-regulation of HSP27 expression will be prepared.

b.HSP27 gene expression will be a) downregulated in ER+ MCF7 cells by transformation with antisense gene constructions and b) expressed independently of estrogen in ER negative MDA-231 cells. Because of difficulties in obtaining satisfactory stable cell lines, this will be achieved using the adenovirus infection/expression system. Cells expressing different levels of HSP27 will be compared with respect to proliferation rate, general motility, chemotactic properties, and resistance to general stress or anti-proliferative drugs.

c. The importance ofHSP27 phosphorylation for the effects observed in Aim 2 will be studied by transforming MDA-231 cells with genes encoding non-phosphorylatable variants of theHSP27 gene.

PROGRESS REPORT

I. ACADEMIC: During the current year, Donna focused on her research. She presented her results at the annual CMB Program Research Meeting at Granlibakken, CA, in November, 1995. Donna attended the Cold Spring Harbor Meeting on Molecular Chaperones and the Heat Shock Response, May, 1996. Donna was co-author on a poster presentation entitled "The Importance of Phosphorylation for HSP27 Function". The presentation included heat survival experiments on hamster fibroblast cell lines expressing different forms of human HSP27 that were conducted by Donna.

II. RESEARCH:

The goals for the second year of the fellowship were to complete the selection and characterization of transfected cell lines. Transient expression of the plasmids constructed during the first year of this Fellowship in mammalian cell lines demonstrated that they contained expressible genes. We have been able to develop several stable transfected cell lines that overexpress HSP27 constitutively and inducibly. However, the production of a significant number of lines that express the protein at high levels has proven to be difficult. To develop a more effective way to introduce and express HSP27 genes in mammary cells, Donna has initiated the use of the replication deficient recombinant human adenovirus system and much of the work to be done in subsequent years will use this vector. Since many of the experiments done this past year involved hands-off cell culture for long periods of time, Donna initiated a second project to study the structural/functional properties of the human HSP27 gene promoter. Figures and Figure Legends are in the Appendix section of this report.

A. Transfection of human mammary cell lines:

1. Testing expression of plasmid constructions containing the human HSP27 gene:

We decided to focus initially on the plasmid constructions containing the human HSP27 gene in the sense orientation. Last year we constructed 2 plasmids containing the human HSP27 gene under control of the constitutively expressed SV40 early and human β-actin promoters (pSV2711 and pHβSL1, respectively). Another plasmid contained the gene under control of the metal-inducible sheep metallothionein promoter (pMT2711). In order to confirm that the plasmids were capable of expressing human HSP27, equivalent amounts of each construction was introduced into 023 hamster fibroblasts and the presence of the human protein was determined by Western blot analysis using anti-human HSP27 antibody. As can be seen in Fig. 1, all three plasmids programmed expression of human HSP27. The SV40 promoter appeared to produce a higher level of the human protein than either the β-actin or metallothionein promoters. In this experiment, induction of the metallothionein promoter by treatment with 3 μM $CdCl_2$ increased the level of expression only slightly. These results validated the use of these three plasmids for the development of stable human cell lines expressing elevated levels of HSP27.

2. Optimization of selection conditions:

We compared the use of puromycin with the more commonly used G418 method for selection of stably transfected cell lines. MCF7 and MB-MDA231 mammary tumor cells were maintained in DMEM supplemented with 10% fetal calf serum and 6 ng/ml insulin. Optimal transfection conditions for transient gene expression using the Lipofectin reagent (GIBCO/BRL), described in the previous annual progress report, consisted of exposure to 2 μg of plasmid DNA with 20μl of Lipofectin reagent in Opti-MEM serum-free medium (GIBCO) for 16 hours. Cells were then fed with complete DMEM containing 5% fetal calf serum and 6 ng/ml insulin. Twenty-four hours later, the medium was changed to selection medium containing puromycin (after transfection with the plasmid pPUR (Clontech)), or G418 (following transfection with pSV2neo). Initial experiments to determine levels of intrinsic resistance to puromycin in MCF7 cells showed no survival in puromycin at either 2.5, 5, or 10 μg/ml. Another experiment using lower concentrations was performed to determine the maximum puromycin level that permitted survival of untransfected cells (Fig. 2). This was shown to be 0.31 μg/ml for both MCF7 and MB-MDA231 cells. Similar experiments were performed using G418, and the maximum survivable level for untransfected cells was 0.25 mg/ml for MCF7 cells and 0.5 mg/ml for MB-MDA231 cells (data not shown). It was decided to proceed with puromycin selection since this drug is much more economical and did not show the batch to batch variability in potency seen with G418.

An incubation time of one month is required to obtain good colonies with the MCF7 and MB-MDA231 strains under our growth conditions. In the initial attempt to isolate stable transfectants, we treated cells with a 10:1 ratio of gene-bearing plasmid to pPUR and selected with medium containing 10 μg/ml puromycin, the concentration that we had previously used successfully to select stable overexpressors of human HSP27 in hamster fibroblasts. However, no colonies were obtained in this experiments. Clearly the mammary cells are exquisitely sensitive to puromycin. Subsequently, a concentration of 1 μg/ml puromycin was used for selection. We ultimately obtained the following transformants:

- 3 MCF7 clones transfected with pHβSL1A, a plasmid expressing an antisense transcript of HSP27 driven by the β-actin promoter.
- 35 MB-MDA231 clones transfected with pHβSL1S, a plasmid expressing a sense transcript of HSP27 driven by the β-actin promoter.
- 25 MB-MDA231. clones transfected with pSV2711, plasmid expressing HSP27 from the SV40 promoter.
- 25 MB-MDA231 clones transfected with pMTO2711, a plasmid expressing HSP27 from the sheep metallothionein promoter.

3. Assay for HSP27 expression in puromycin-selected clonal mammary tumor cell lines.

The expression of the antisense sequences in the MCF7 cells will be undertaken after more clones have been obtained. The MB-MDA231 clones have been assayed for overexpression of HSP27 by Western

blot analysis. The first antibody was anti-human HSP27, followed by alkaline phosphatase conjugated rabbit anti-mouse immunoglobulin, and blots were either developed with a colorimetric assay using NBT and BCIP (Boehringer) as substrate, or with a chemiluminescent assay using CDP* (Tropix). The chemiluminescent assay was used for quantitation of expression using a BioRad Phosphoimager. The results showed that 8 of the 35 clones transfected with pHβSL1S constitutively express HSP27 at levels greater than the parent cell line (FIG 3). Clonal lines Hb-4 and Hb-5 contain 7 - 8 fold more Hsp27 than the parent cell line. Only one clone (SV-9) selected from the pSV2711 transfection showed increased expression of HSP27. However, the level of HSP27 expression in this clone is greater than 10-fold higher than control. We are disappointed that only one of the 25 clones transfected with the SV40 promoter construction showed elevated expression of HSP27. In order to avoid misinterpreting data that may be the result of clonal variation between cell lines rather than expression of HSP27, we are screening for additional high expressing clones before beginning the experimental analysis.

Prior to screening for metal-inducible expression of HSP27 in clonal lines transfected with the sheep metallothionein promoter plasmid, we determined the optimal concentration of cadmium to be used for induction. The appropriate concentration should be lower than that which induces a stress response in order to distinguish the effects of HSP27 expression from the effects of inducing the endogenous heat shock protein genes. MB-MDA231 cells were plated 16 hours before assay in 6-24 well plates. Cadmium chloride was introduced at concentrations from 0.25 to 10μM in fresh DMEM + 10% fetal calf serum. Twenty-four hours later, the treated cells and an untreated control culture were lysed and analyzed by Western blotting with anti-HSP27 and anti-HSP70 as described above. FIG 4 shows that increased expression of endogenous Hsp70 is not detectable below 2.0 μM cadmium.

Twelve clonal lines transfected with pMTO2711 have been screened and only 6 showed elevated HSP27 expression following incubation in 3 μM cadmium . While this concentration of metal caused a slight induction of Hsp70, no significant induction of the endogenous HSP27 gene is produced (see FIG 4). When these 6 clones were screened again in the presence and absence of 3 μM cadmium, only clones 3, 5, 6 , and 7 showed increased HSP27 expression in the presence of the metal (FIG 5). Induction was only approximately 2-fold. The time course of HSP27 accumulation following addition of 3 μM cadmium is shown in FIG 6. In this experiment, maximum accumulation of HSP27 is apparent 12 - 14 hr following addition of the metal. In this experiment induction was approximately 3 -fold higher than control.

B. Establishment of the replication deficient adenovirus system for overexpressing hsp27 in mammary cell lines:

We now have established several clonal cell lines that overexpress HSP27, yet we are disappointed by the relatively low level of expression seen in most of the clones. These results obtained with human mammary tumor cells are in contrast to our previous studies in rodent fibroblasts. There we found it to be relatively easy to obtain large numbers of clones expressing levels of human HSP27 at least 10-fold greater than the endogenous small heat shock protein. Furthermore, the mammary tumor clones we obtained using the metallothionein promoter are quite leaky and show only a low level of induction. The possibility of induction of the endogenous heat shock proteins by cadmium treatment adds an additional complication to the study of the effects of HSP27 expression on tumor cell properties. The possibility of clonal variation

between cell lines that may be unrelated to HSP27 levels might further limit their usefulness. After considering all these factors, we have decided to concurrently pursue an alternative method for altering the level of HSP27 in tumor cells: infection with recombinant adenovirus vectors containing sense, anti-sense, and mutant forms of the HSP27 gene (11).

In a collaborative project with Jodie Martin and Wolfgang Dillman at UC San Diego, we have constructed recombinant adenovirus containing the wild type human HSP27 gene in the sense and anti-sense orientation with respect to the powerful CMV promoter. In addition, we have also introduced various mutations that affect phosphorylation into the viral-contained gene. The purpose of constructing these vectors was to study the effects of HSP27 expression in cardiac tissue with respect to resistance to ischemic damage. Infection of rat cardiomyocytes with these recombinant adenoviruses at multiplicities of infection between 3 and 10 results in a substantial induction of the various forms of HSP27. This work has now been presented at several meetings. We now plan to use these vectors to program HSP27 levels in MCF7 and MB-MDA231 cells.

The advantages of the adenovirus system are several. Infection results in rapid transient induction of the protein with maximum levels typically achieved within 48 hr. In non-permissive cells, no other adenovirus genes are expressed. Unlike other transient gene expression systems, a very high uniform population of expressing cells (greater than 90%) can be achieved. Currently, Donna is determining the multiplicity of infection required for high level expression of HSP27 in MCF7 cells and MB-MDA231 cells. We anticipate that the level of expression of HSP27 that will be obtained using the adenovirus vectors will be higher than that seen with the stable transfected cell lines. At the very least, the adenovirus system will allow us to test our hypothesis that HSP27 levels affect tumor cell growth properties using an independent experimental approach.

C. Characterization of HSP27 promoter activity in mammary tumor cell lines.

The selection of clonal cell lines involves a significant amount of waiting, and during this time Donna has initiated a new series of studies to define the promoter elements of the HSP27 gene that are responsible for the observed difference in expression of the protein in estrogen receptor positive cells such and MCF7 and estrogen receptor negative cells such as MB-MDA231. She is using constructions in which truncated segments of the HSP27 promoter drive expression of a luciferase reporter gene. These constructions have already been prepared in our laboratory as part of a collaborative study with Drs. Stephanie Oesterreich and Suzanne Fuqua at UT San Antonio. Using transient gene expression, Donna has shown that the HSP27 promoter driven reporter gene is more efficiently expressed in MCF7 cells than in MB-MDA231 cells, both constitutively and following heat stress (FIG 7A&B). She has shown that deletion of the single consensus heat shock element (GAANNTTC) essentially eliminates heat inducibility. However, the constitutive transcription from the promoter, which is 20-fold greater in MCF7, is unaffected by deletion of the heat shock element. Thus, other sequences contained in the HSP27 promoter are responsible for this phenomenon. Donna hopes to identify the relevant control elements by further mutational analysis of the promoter segment. Understanding the mechanistic basis for differential expression of stress proteins in different tumor cell populations is directly relevant to the breast cancer problem. Growth conditions found in a solid tumor are known to induce the stress response. The differential ability of tumors to mount a stress response may expose new avenues for therapeutic intervention. Nonetheless, Donna considers this a side project and her pursuit of this problem should not detract from her focus on the proposed thesis objectives.

CONCLUSIONS

Donna has had to develop flexibility in order to successfully move forward in her research. The results of the past year have been instructive with regard to the difficulties of extrapolating from results of experiments performed in diverse cell types. The establishment of stable clonal cell lines for over- or underexpression of HSP27 has been more difficult to accomplished than was anticipated. However, an alternative methodology using adenovirus vectors is available and is being evaluated. In addition, Donna has initiated a study of the function of the HSP27 gene promoter in response to heat in MCF-7 and MDA231 cells.

In the coming year we expect to obtain more stable cell lines overexpressing HSP27, in which it will be possible to determine the effect of HSP27 levels on mammary tumor cell proliferation rate. In addition, we will use replication deficient adenovirus vectors for modulating HSP27 levels in mammary tumor cell lines. These transformants can be used for the studies of HSP27 effects on motility, and on cell resistance to hyperthermia and drugs.

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APPENDIX

Fig. 1 Transient expression of human Hsp27 in the 023 hamster cell line.

Hamster 023 fibroblasts were transfected with 2 μg of the indicated plasmid DNA using 200 μl of Lipofectin for 6 hours. At 24 hours post transfection, medium containing 3 μM $CdCl_2$ was added to half of the cells transfected with pMT2711, in order to induce expression of Hsp27 from the metallothionein promoter. At 48 hours post transfection, the cells were harvested, and 4 μg of protein from each sample was analyzed by SDS-PAGE and Western blot. The blot was probed with mouse anti-human Hsp27 monoclonal antibody followed by alkaline phosphatase conjugated goat anti-mouse IgG, and developed with NBT and BCIP.

Transient expression of human Hsp27 in a hamster cell line

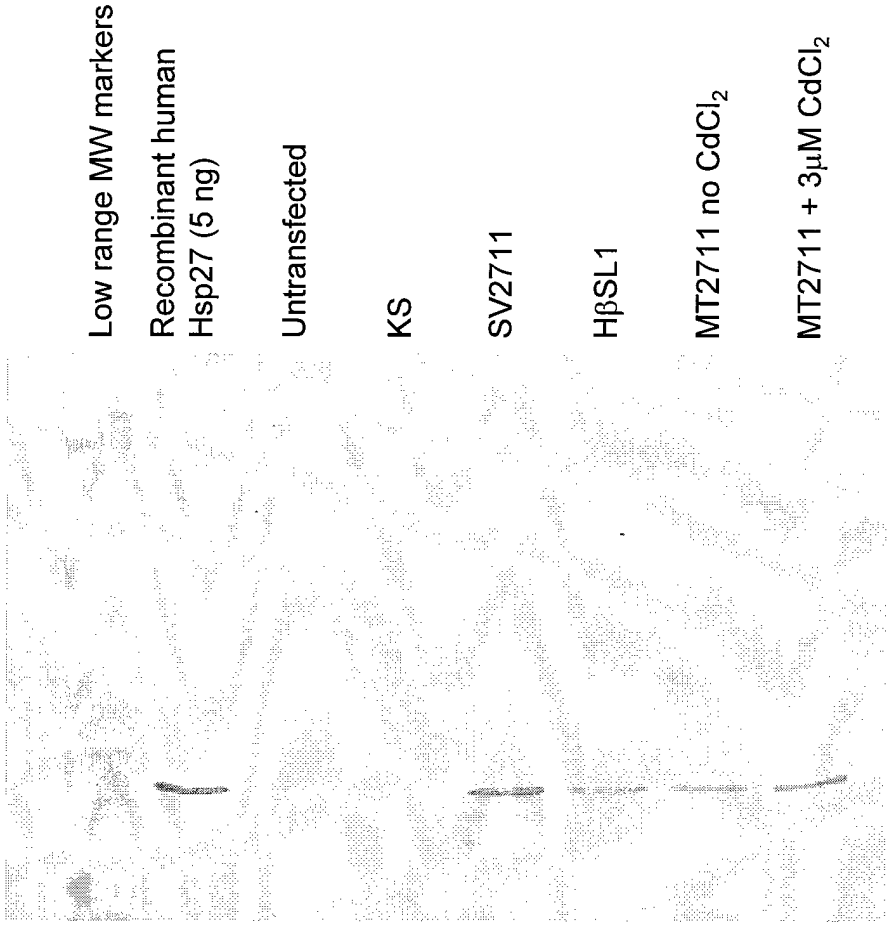


Fig. 2 Survival of MCF7 and MDA231 cell lines in puromycin.

Cells were plated at a density of 3 X 10^4 cells per well in DMEM media supplemented with 10% fetal calf serum and 6 ng/ml insulin. Puromycin at the indicated concentration was added 24 hours after plating. The cultures were incubated at 37^0 C in an atmosphere of 5% CO_2 for 1 week. To visualize surviving cells, the wells were rinsed in PBS and stained with Coomasie Blue.

Survival of mammary tumor cell lines in puromycin

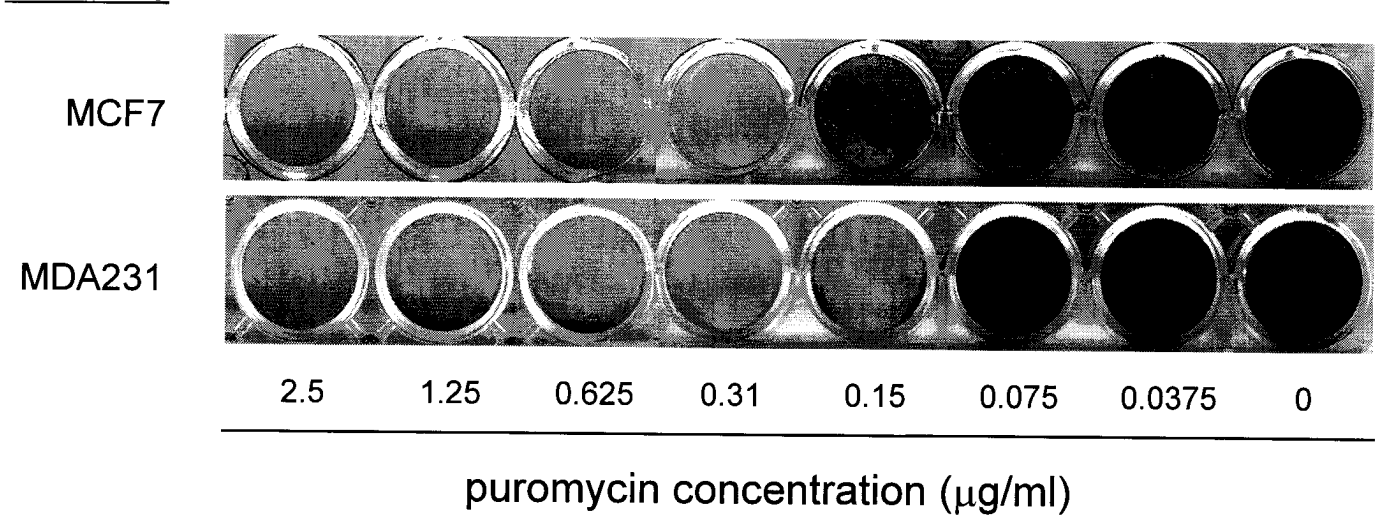
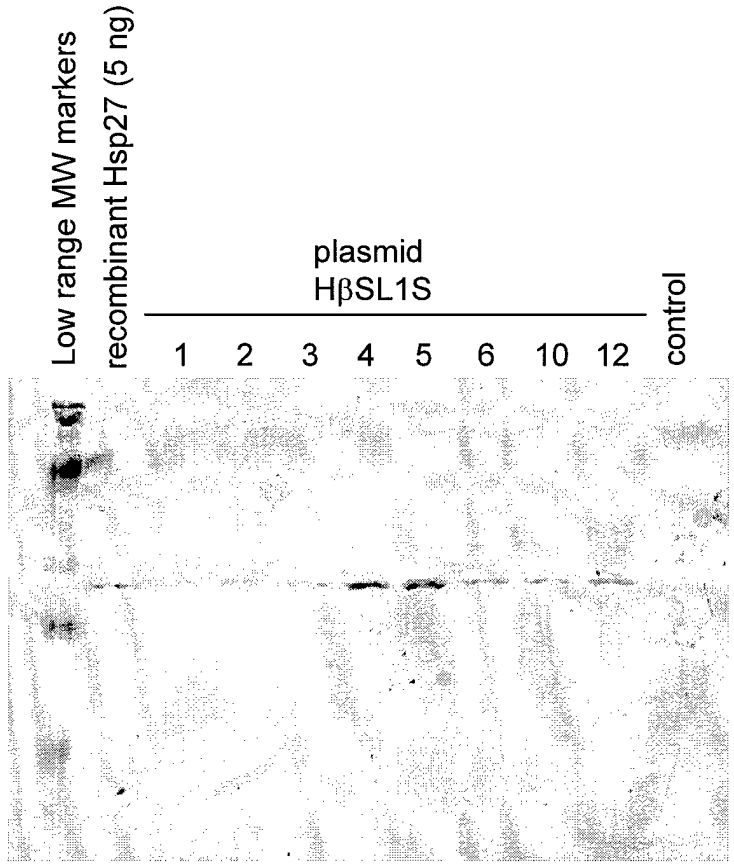


Fig. 3 Expression of Hsp27 in puromycin-selected MDA231 clones.

Two 6 well plates of MDA231 cells were seeded at 2 X 10^5 cells per 35mm well. After 24 hours, cells were transfected with 1 ug total DNA using 10 µl Lipofectin per well for 6 hours. Cells were cotransfected with a 1:10 ratio of pPUR to plasmid (KS, pSV2711, or pHβSL1S.) After 60 hours, media containing 1 µg/ml puromycin was added to the wells and cells were left undisturbed for 2 weeks. Small colonies were counted, trypsinized, and the cells grown as pooled transformants. For cloning, pooled transformants were plated at 30 cells per 100mm dish, and colonies were isolated after 14 days. Each clone was plated into a 35mm well, expanded to a T75 culture over a 6 week period. Prior to cryogenic storage, cell lysates were prepared for protein analysis. The protein concentration was determined in each lysates and equal amounts of protein (10 µg) were analyzed for HSP27 content by Western blotting.

Expression of Hsp27 in stable MDA231 clonal cell lines



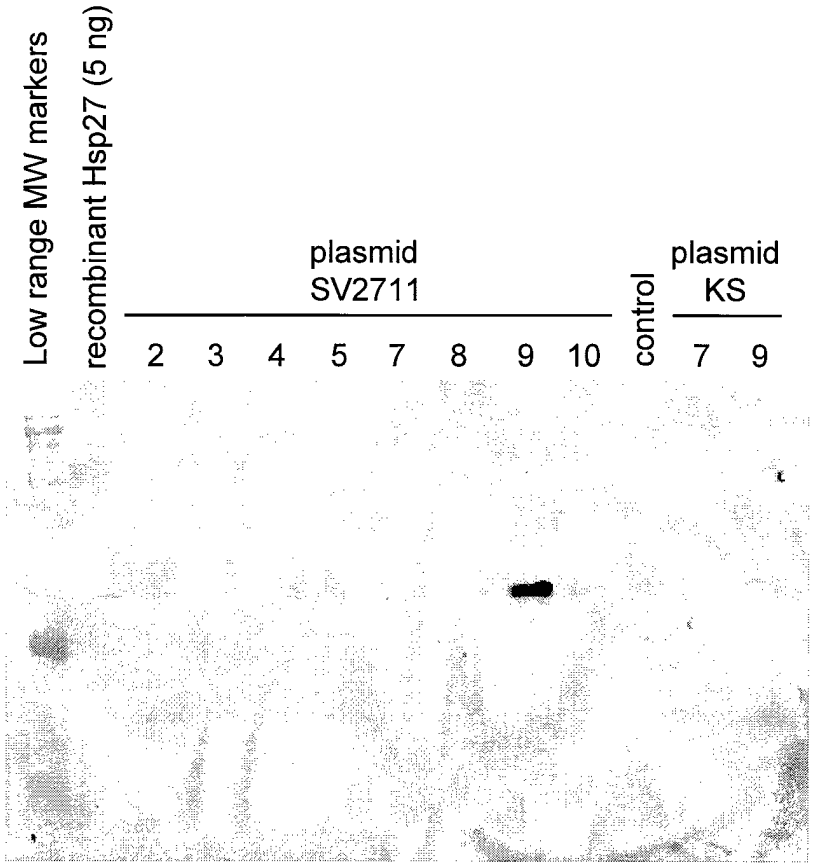


Fig 4. Effect of CdCL2 concentration on endogenous Hsp27 and Hsp70 levels in MB-MDA231 cells.

Cells were plated at a density of 1.2 X 10^5 per 35mm well. After 24 hours, cells were fed with media containing $CdCl_2$ at the indicated concentrations. Cells remained in this media for 24 hours, at which time they were lysed, protein concentrations were determined, and equal amounts of protein (10 μg) were analyzed for Hsp27 and inducible Hsp70 by Western blotting.

Effect of $CdCl_2$ concentration on endogenous Hsp27 and Hsp70 levels in MDA231

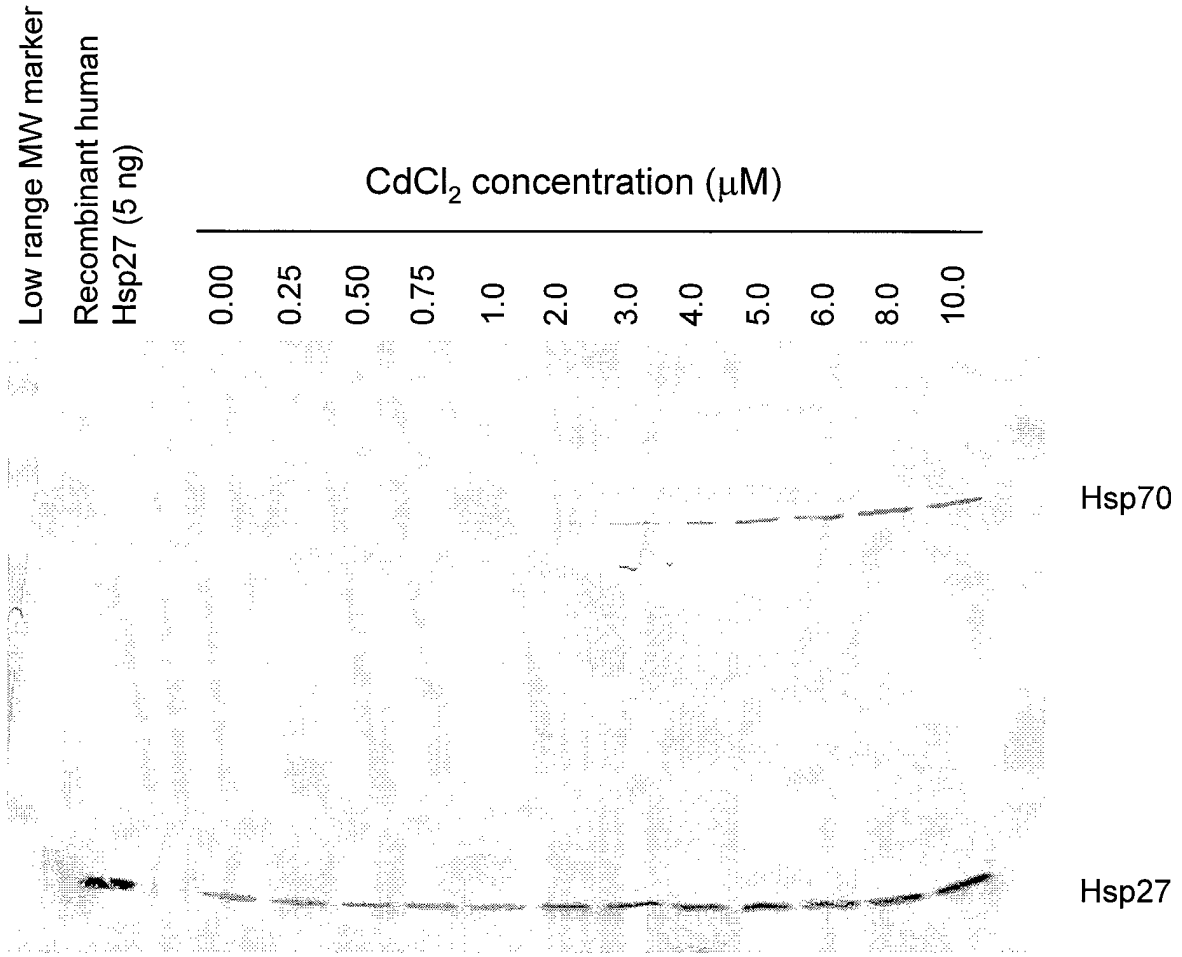


Fig. 5 Induction of Hsp27 and inducible Hsp70 in MB-MDA231 clones in response to $CdCl_2$.

Clones were isolated by cotransfected with 0.2 mg pPUR plasmid (for selection) and 1.8 mg pMT2711 in 6 well plates, using 20 µl Lipofectin for 6 hours. Fresh media containing 1 µg/ml puromycin was added to the cells after 48 hours, and colonies were allowed to form for 2 weeks. Individual colonies were then trypsinized, expanded, and analysed for expression of Hsp27 in the presence of 3 µM $CdCl_2$ for 24 hours. Clones expressing higher than control levels of Hsp27 were selected. These clones were then plated in two sets of 6 well plates at 2 X 10^5 cell per 35mm well. After 24 hours one set of plates was treated with 3 µM $CdCl_2$ and the other was fed with regular media. The cells were then lysed after 24 hours and the protein concentrations were determined. Equal amounts of protein (10 µg) were analyzed for Hsp27 and inducible Hsp70 content by Western blotting.

Induction of Hsp27 and inducible Hsp70 in MDA231 clones in response to $CdCl_2$

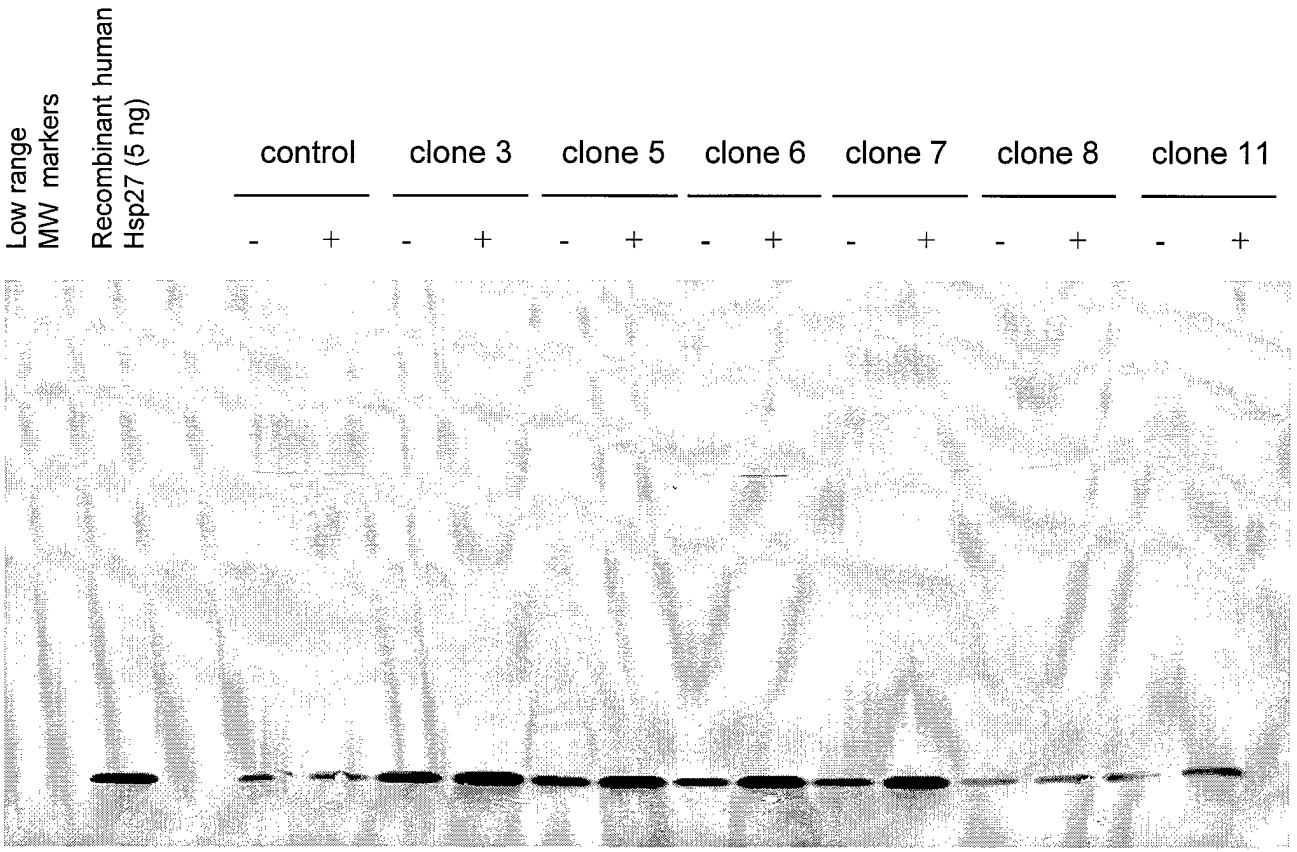


Fig. 6 Time course of Hsp27 induction by 3 μM $CdCl_2$ in MB-MDA231 MT2711 clone #5.

Cells were grown in selection-free media for 4 days, trypsinized, and seeded in 6 well plates at a density of 2 X 10^5 per 35mm well. At 24 hours, a zero time sample was collected and growth medium containing 3μM $CdCl_2$ was added to the remaining cells. Over the next 24 hours, cells were collected at the indicated intervals. Protein concentrations were determined, and equal amounts of protein (10 μg) were analyzed for Hsp27 content by Western blotting.

Time course of Hsp27 induction by $CdCl_2$ in MDA231 MT27 clone #5

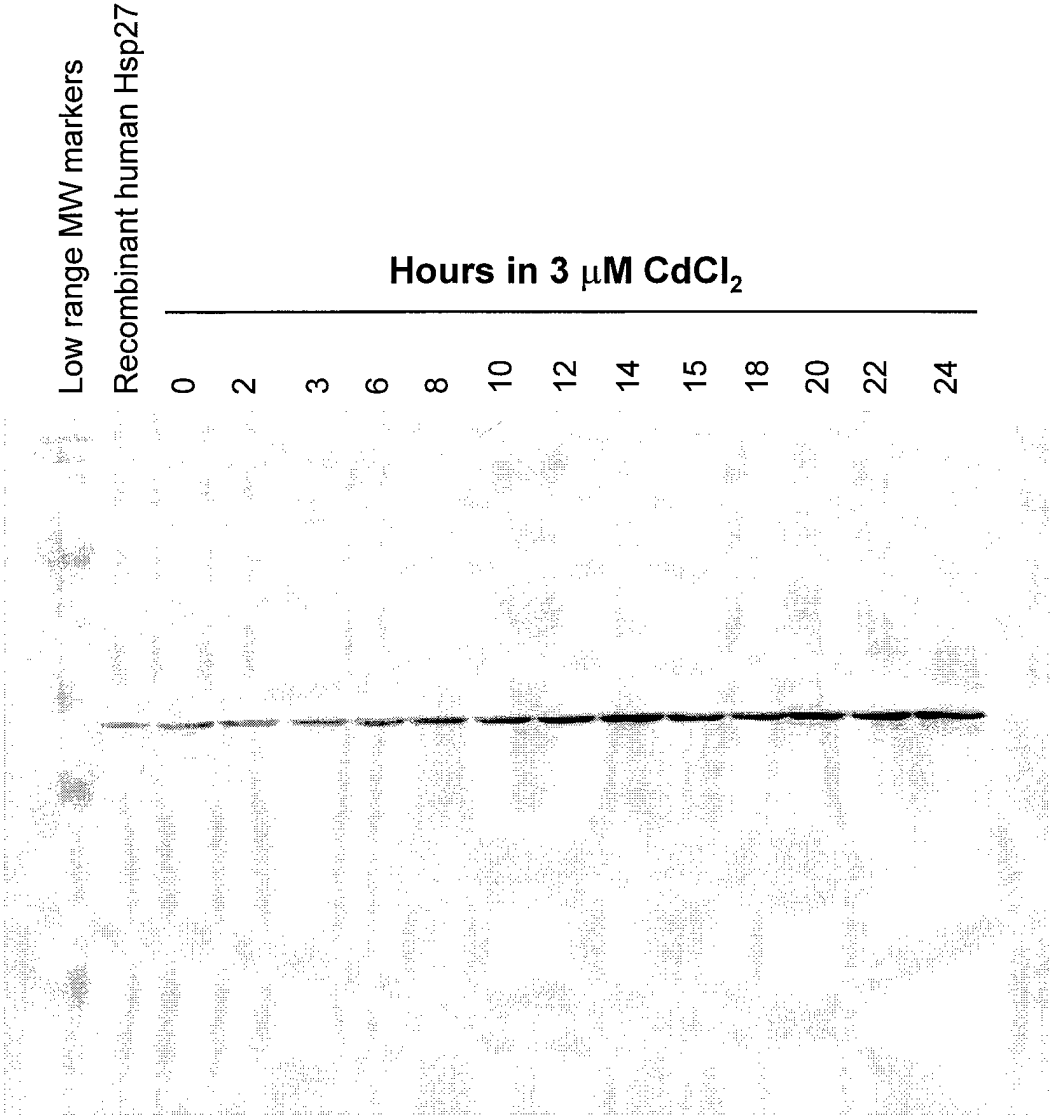


Fig. 7 Hsp27 promoter deletion luciferase constructs.

Plasmids were constructed containing deletion mutants of the Hsp27 promoter driving a luciferase reporter gene. BglLux contains 1057 base pairs of upstream sequence and is considered the full length promoter. BglLuxMut8 contains the same region as BglLux, with a deletion of the heat shock element (HSE) at position -124. The BamLux, PstLux, and EcoLux constructions contain 688, 410, and 176 base pairs of upstream sequence respectively. Half ERE is one half of the consensus palindromic estrogen response element.

Hsp27 promoter deletion luciferase constructs

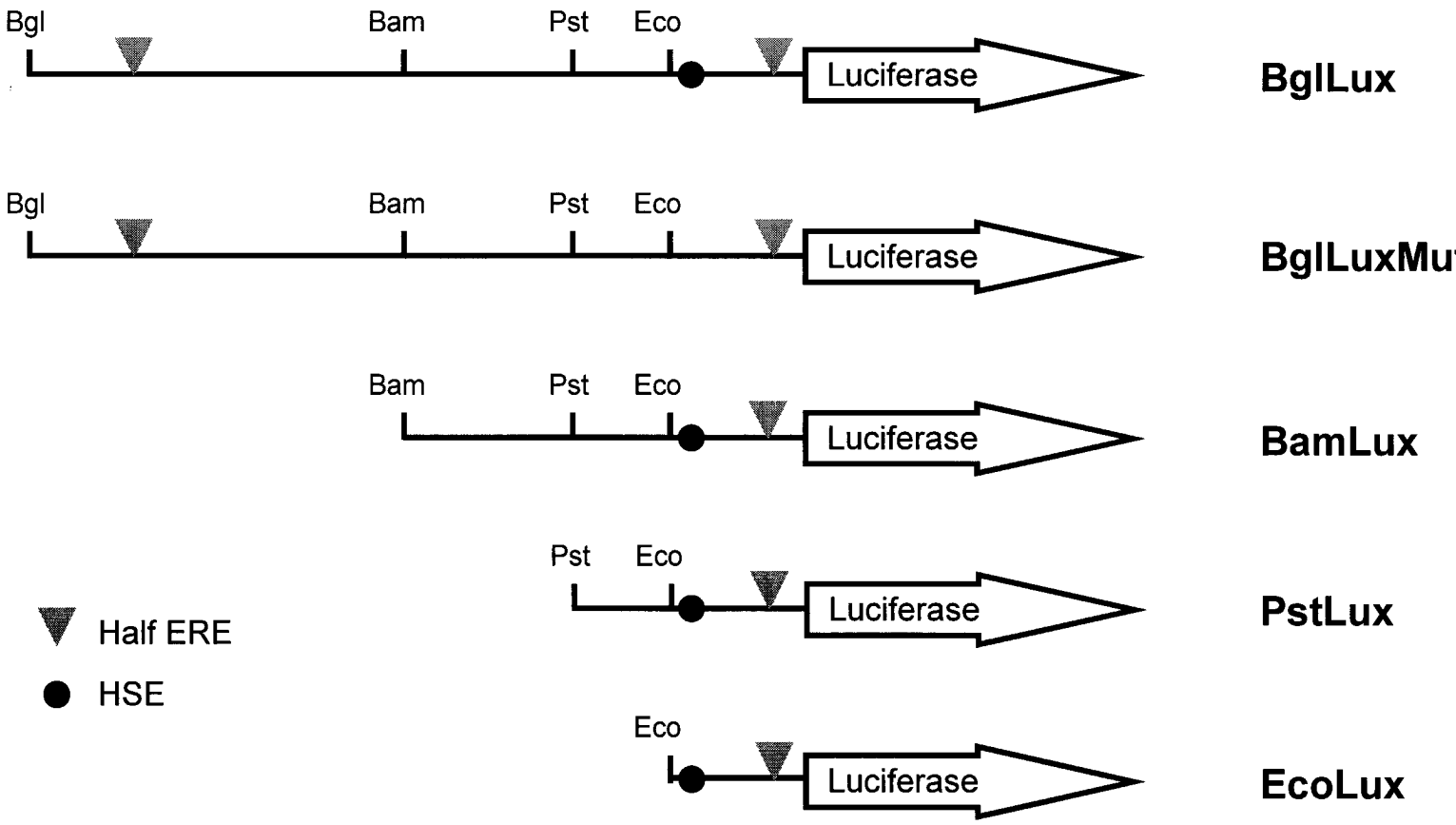
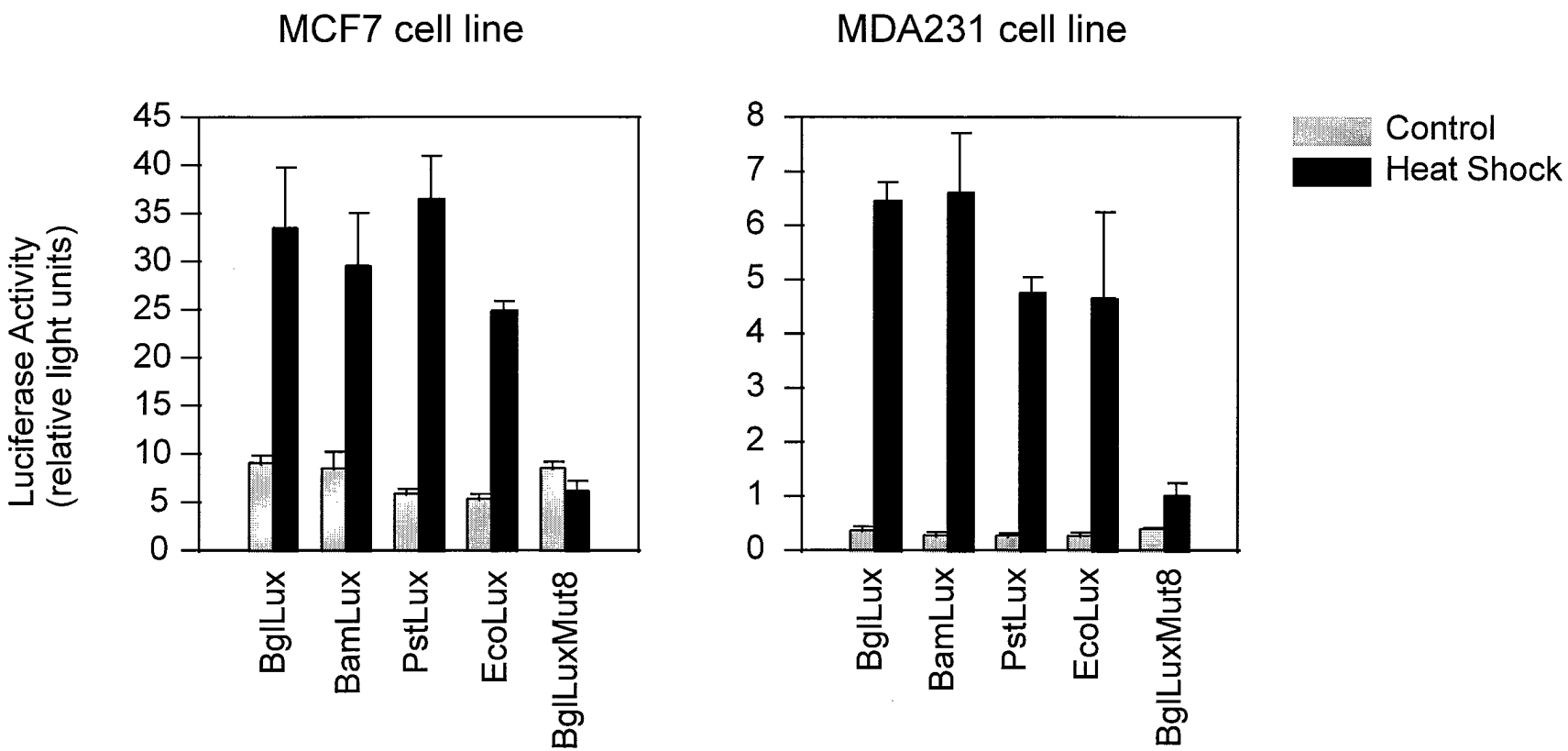


Figure 8. Heat induction of Hsp27 promoter deletion constructs in MCF7 and MB-MDA231 cell lines.

Cells were plated at a density of 2 X 10^5 per well in 35 mm wells and allowed to grow for 24 hours. Cells were transfected with 1.5 μg of total DNA, consisting of 0.75 μg of pSVβGal, 0.25 μg of Hsp27 promoter construct, and 0.5 μg of Bluescript KS plasmid. Transfection was performed using 10 μl LipofectAMINE per well for 5 hours. After 24 hours of recovery, the cells in each well were split evenly into two new wells and allowed to grow an additional day. Half the cultures were heat treated at 42°C for 1 hour and allowed to recover for 3 hours at 37°C. All the cells were then lysed and analysed for luciferase and β-galactosidase activity. Note the difference in scale of the y-axes.

Heat induction of Hsp27 promoter deletion constructs in mammary cell lines



ABSTRACT

THE IMPORTANCE OF PHOSPHORYLATION FOR HSP27 FUNCTION

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The human small heat shock protein, HSP27, is phosphorylated by MAPKAP kinase 2 on three serine residues following heat shock, other environmental stresses, and in response to mitogens and cytokines. Elevated levels of HSP27 confer increased resistance to thermal killing and drug toxicity. In addition, expression of wild type or non-phosphorylatable mutant forms of HSP27 has pronounced effects on the actin cytoskeleton. The importance of phosphorylation for HSP27 function is controversial. With J. Landry's group, we have shown that a nonphosphorylatable mutant form of HSP27 does not confer significant heat resistance in CCL39 hamster fibroblasts. M. Gaestel and coworkers, in contrast, found increased thermoresistance in ascites cells expressing either wild type or nonphosphorylatable HSP27.

We have now studied the importance of HSP27 phosphorylation for heat resistance in a number of clones derived either from CCL39, an attachment-dependent hamster fibroblast line, and its tumorigenic variant, 023. We confirm that mutant forms of HSP27 (either ser->ala, ser->gly or ser->asp mutants) do not provide a significant increase in heat resistance in CCL39 cells. However, in 023 cells, both the mutant and wild type forms of HSP27 increase heat resistance. Despite the absence of an effect on survival, phosphorylation of HSP27 modulates actin-dependent cellular functions in 023 cells. In particular, we find that overexpression of wild type hsp27 increases serum-stimulated motility of 023 cells, while the non-phosphorylatable variants of HSP27 have no effect.

HSP27 has chaperone activity in vitro that appears to be phosphorylation independent, and an actin-modulating function(s) that involves phosphorylation. Depending on cell type, the chaperone function or actin modulating function of HSP27 may be of greater importance for conferring stress resistance. Our results suggest that phosphorylation-dependent stabilization of F-actin by HSP27 may only be important for increased thermal resistance in attachment dependent cells.